Clones Identification and Genetic Characterization of Garnacha Grapevine by Means of Different PCR-Derived Marker Systems

Stefano Meneghetti · Angelo Costacurta · Enrica Frare · Graziana Da Rold · Daniele Migliaro · Giacomo Morreale · Manna Crespan · Vicente Sotés · Antonio Calò

Abstract This study uses PCR-derived marker systems to investigate the extent and distribution of genetic variability of 53 Garnacha accessions coming from Italy, France and Spain. The samples studied include 28 Italian accessions (named Tocai rosso in Vicenza area; Alicante in Sicily and Elba island; Gamay perugino in Perugia province; Cannonau in Sardinia), 19 Spanish accessions of different types (named Garnacha tinta, Garnacha blanca, Garnacha peluda, Garnacha roja, Garnacha erguida, Garnacha roya) and 6 French accessions (named Grenache and Grenache noir). In order to verify the varietal identity of the samples, analyses based on 14 simple sequence repeat (SSR) loci were performed. The presence of an additional allele at ISV3 locus (151 bp) was found in four Tocai rosso accessions and in a Sardinian Cannonau clone, that are, incidentally, chimeras. In addition to microsatellite analysis, intravarietal variability study was performed using AFLP, SAMPL and M-AFLP molecular markers. AFLPs could discriminate among several Garnacha samples; SAMPLs allowed distinguishing few genotypes on the basis of their geographic origin, whereas M-AFLPs revealed plant-specific markers, differentiating all accessions. Italian samples showed the greatest variability among themselves, especially on the basis of their different provenance, while Spanish samples were the most similar, in spite of their morphological diversity.

Keywords Cannonau · Grenache · AFLP · M-AFLP · SAMPL · Intravarietal variability

Introduction

The great morphological and genetic variability characterizing the genus *Vitis* is the main problem for grapevine cultivars identification and gives rise to confusion and ambiguity. These technical difficulties are particularly evident in the case of varieties that have been cultivated for centuries and are widely distributed. The Garnacha grapevine is one of the most widely planted in the world (240,000 ha) [1]. It is cultivated in Southern France, Spain, Italy, South America, Australia and California, where is known with different local names such as Garnacha in Spain, Grenache in France, Australia and California; and Cannonau, Alicante and Tocai rosso in Italy [1]. The first Garnacha description in Spain belongs to Alonso de Herrera (1513) who named this variety "red of Aragon" [2]. In addition to historical reports, the likely Spanish origin of Garnacha is suggested also by ampelographical evidence. Currently, the Garnacha covers around 77,132 ha in the principal vitivinicultural Spanish areas (Registro vitícola español 2009) and despite its prevalence in nearby Navarra and Catalonia, Garnacha was not widely planted in the Rioja till the early twentieth century, when vineyards were replanted following the phylloxera disease [3]. An alternative theory has the Italian island of Sardinia (where the grape is known as Cannonau) as the possible originating source

when the vine being introduced to Spain probably in the fourteenth century [4]. According to this theory, Garnacha came to Spain between the thirteenth and fifteenth century when Sardinia was part of the Aragon kingdom and interchange of grapevine species was possible [4]. Finally, Garnacha was spread to France from Aragón probably at the end of 1700 [5].

Outside the European borders, Grenache was one of the first varieties to be brought in Australia in the eighteenth century and became the most widely planted red wine grape variety in that country, until the diffusion of Syrah in the mid-1960s [6]. In the nineteenth century, California wine growers prized the vine's ability to produce high yields and withstand heat and drought conditions; hence it was extensively planted throughout the warm San Joaquin Valley. In the early twentieth century, Grenache was one of the first *Vitis vinifera* L. grapes to be successfully vinified during the early development of the Washington wine industry [7].

Many morphological and molecular markers have been used for the characterization of grapevine *Vitis vinifera* L. germplasm. Among these, ampelographic characterization according to morphological features has been useful in the identification of well-known grape varieties and has facilitated the clarification of ambiguous denominations and the establishment of phenological relationships [8]. Nevertheless, the morphological characteristics can be affected by the environment [9] and generally, it is not possible to distinguish very close genotypes, such as clonal selections derived from a variety [10]. The intra-varietal variability in *Vitis vinifera* is the consequence of two different phenomena: (a) the presence of different sub-varieties, observed only in some cultivars, and (b) the clonal variability. This variability in a specific cultivar also includes two different levels, the morphological variability and the genetic variability [10]. Biotypes identification has traditionally been solved using ampelography, ampelometry and chemical traits analysis but these tools have also resulted in several false attributions, in particular when used at the clonal level [11].

Isozymes, based on variability at proteins level [12], have frequently been used for these purposes [13–15] with different results depending on the genetic relationships among the materials analysed [16–18]. For these reasons, the DNA molecular analyses [19] with ampelography, ampelometry and chemistry, are essential for precise grapevine identification and also, to investigate the genetic differences among *Vitis vinifera* L. clones [20, 21]. Usually grapevine accessions of the same cultivar showed one specific microsatellite profile [10], but in some cultivar these simple sequence repeat (SSR) markers can also discriminate among genotypes by microsatellite mutations [10, 22].

Many molecular markers have been used for the differentiation of clones and these include, inter-microsatellites (I-SSR) [23], random amplified polymorphic DNA (RAPD) [24, 25], amplified fragment length polymorphism (AFLP) [26–28], selective amplification of microsatellite polymorphic loci (SAMPL) [29], single nucleotide polymorphism (SNP) [30], specific sequence amplified polymorphism (S-SAP) [31], methyl-sensitive amplified length polymorphism (M-SAP) [32], inter-retrotransposon amplified polymorphism (IRAP) and retrotransposon-microsatellite amplified polymorphism (REMAP) [33, 34], chloroplast DNA polymorphisms [35], microsatellites amplified fragment length polymorphism (M-AFLP) [10, 11, 36].

Some studies at morphological level have showed differences among French Grenache, Italian Tocai rosso and Italian Cannonau [37] and also molecular preliminary studies using AFLP-based markers [38]. Nevertheless, a complete clonal characterization of accessions from different origins has never been made.

In this study, genetic variability of Italian, Spanish and French Garnacha was investigated using AFLP, M-AFLP, SSR and SAMPL molecular markers [39]. The purpose of this project was to study the utility of these marker types in the Garnacha genotypes (clones) discrimination and about the quantification of molecular variability of this important grapevine cultivar.

Materials and Methods

Fifty-three Garnacha accessions were investigated: 28 Italian accessions which six Tocai rosso (called TOR) from the Vicenza area, eight Alicante (called ALI) from Sicily and Elba island, four Gamay perugino (called GAM) from Perugia province, and 10 Cannonau (called CAN) from Sardinia; 19 Spanish accessions (called GAR) named Garnacha tinta, Garnacha blanca, Garnacha peluda, Garnacha roja, Garnacha erguida and Garnacha roya; six French accessions (called GRE) named Grenache and Grenache noir (Table 1).

DNA Extraction

The DNA was extracted according to the method described by Meneghetti et al. [40] from the young leaves of the 53 Garnacha accessions. Genomic DNA was stored undiluted in TE (10 mM Tris–HCl, 1 mM EDTA) buffer pH 8.0 at −20°C [41]. An aliquot of genomic DNA was also assayed by electrophoresis on 1% agarose gels [41]. The DNA was quantified on the base of fluorometric determination using the PicoGreen dsDNA quantitation assay (Invitrogen) by the FLx800 TBI Fluorometer (Bio-Tek) [39].

Table 1 Name and geografic origins of 53 Garnacha genotypes with the accession codes

Code*	Accession name	Note	Geographic origin
ALI-1	ALICANTE	Biotype 4.2	Italy—Elba island
ALI-2	ALICANTE	Biotype 1	Italy—Sicily (Catania)
ALI-3	ALICANTE	Biotype 2	Italy—Sicily (Catania)
ALI-4	ALICANTE	Biotype 3	Italy—Sicily (Catania)
ALI-5	ALICANTE	Biotype 4	Italy—Sicily (Catania)
ALI-6	ALICANTE	Biotype 5	Italy—Sicily (Catania)
ALI-7	ALICANTE	Biotype 6	Italy—Sicily (Catania)
ALI-8	ALICANTE	Biotype 7	Italy—Sicily (Catania)
CAN-9	CANNONAU 1D	Preselection Biotype	Italy—Sardinia (Jerzu)
CAN-10	CANNONAU 4C	Preselection Biotype	Italy—Sardinia (Jerzu)
CAN-11	CANNONAU 3C	Preselection Biotype	Italy—Sardinia (Jerzu)
CAN-12	CANNONAU 6C	Preselection Biotype	Italy—Sardinia (Jerzu)
CAN-13	CANNONAU**	Clone CAP VS5	Italy—Sardinia (Nuoro)
CAN-14	CANNONAU	Preselection Biotype	Italy—Sardinia (Nuoro)
CAN-15	CANNONAU	Preselection Biotype	Italy—Sardinia (Nuoro)
CAN-16	CANNONAU	Preselection Biotype	Italy—Sardinia (Nuoro)
CAN-17	CANNONAU	Preselection Biotype	Italy—Sardinia (Nuoro)
CAN-18	CANNONAU	Preselection Biotype	Italy—Sardinia (Nuoro)
GAM-19	GAMAY PERUGINO	Clone 1 ISV-ICA-PG	Italy—Umbria (Perugia)
GAM-20	GAMAY PERUGINO	Preselection Biotype	Italy—Umbria (Perugia)
GAM-21	GAMAY PERUGINO	Preselection Biotype	Italy—Umbria (Perugia)
GAM-22	GAMAY PERUGINO	Preselection Biotype	Italy—Umbria (Perugia)
TOR-23	TOCAI ROSSO**	Clone ISV-C VI 17	Italy—Colli Berici (Vicenza)
TOR-24	TOCAI ROSSO**	Clone ISV-C VI 2	Italy—Colli Berici (Vicenza)
TOR-25	TOCAI ROSSO**	Clone ISV-C VI 3	Italy—Colli Berici (Vicenza)
TOR-26	TOCAI ROSSO**	Preselection Biotype	Italy—Colli Berici (Vicenza)
TOR-27	TOCAI ROSSO	Preselection Biotype	Italy—Colli Berici (Vicenza)
TOR-28	TOCAI ROSSO	Preselection Biotype	Italy—Colli Berici (Vicenza)
GRE-29	GRENACHE	Clone 70 ENTAV	France
GRE-30	GRENACHE	Clone 516 ENTAV	France
GRE-31	GRENACHE	Clone 139 ENTAV	France
GRE-32	GRENACHE	Clone 134 ENTAV	France
GRE-33	GRENACHE NOIR	INRA collection	France
GRE-34	GRENACHE NOIR	INRA collection	France
GAR-35	GARNACHA TINTA	Clone VCR 23	Spain—Aragón
GAR-36	GARNACHA BLANCA	12.260 preselection Biotype	Spain—Cataluña
GAR-37	GARNACHA PELUDA	18.140 preselection Biotype	Spain—Madrid
GAR-38	GARNACHA ROJA	18.048 preselection Biotype	Spain—Castilla y León
GAR-39	GARNACHA TINTA	Clone 16.048	Spain—Castilla y León
GAR-40	GARNACHA TINTA	Clone 16.051	Spain—Castilla y León
GAR-41	GARNACHA TINTA	Clone 6.064	Spain—Andalucia
GAR-42	GARNACHA TINTA	17.055 preselection Biotype	Spain—Castilla y León
GAR-43	GARNACHA TINTA	ARA 4 preselection Biotype	Spain—Aragón
GAR-44	GARNACHA TINTA	ARA 3 preselection Biotype	Spain—Aragón
GAR-45	GARNACHA TINTA	ARA 2 preselection Biotype	Spain—Aragón
GAR-46	GARNACHA BLANCA	2.9 preselection Biotype	Spain—Aragón
GAR-47	GARNACHA ERGUIDA	2.4 preselection Biotype	Spain—Aragón
GAR-48	GARNACHA ROYA	14.11 preselection Biotype	Spain—Aragón

Table 1 continued

Code*	Accession name	Note	Geographic origin
GAR-49	GARNACHA PELUDA	B. A. preselection Biotype	Spain—Aragón
GAR-50	GARNACHA TINTA	31.14 preselection Biotype	Spain—Aragón
GAR-51	GARNACHA TINTA	18.7 preselection Biotype	Spain—Aragón
GAR-52	GARNACHA TINTA	27.13 preselection Biotype	Spain—Aragón
GAR-53	GARNACHA TINTA	6.12 preselection Biotype	Spain—Aragón

* Used for figures and tables where *ALI* Alicante, *CAN* Cannonau, *GAM* Gamay perugino, *TOR* Tocai rosso, *GRE* French Grenache, *GAR* Spanish Garnacha; ** triallelism at ISV3 locus (+151 bp)

SSR Analysis

In order to verify the varietal identity of the studied accessions, SSR analysis was performed. Fourteen microsatellite loci were analysed [41]: the 6 core loci selected within Genres 081 European Project (VVS2, VVMD5, VVMD7, VVMD27, VrZAG62 and VrZAG79), ISV2, ISV3 and ISV4 [42], VVMD24, VVMD26, VVMD28 [43], VMCNG4b9 and VMC2H9 (Vitis Microsatellite Consortium).

The microsatellite PCR mixture (25 µl final volume) was performed by Starlet liquid handling workstations (Hamilton) and contained: 10 ng total DNA, 10 µl of Eppendorf Taq-dNTPs Mix (containing dNTPs, Taq DNA polymerase and specific enzyme buffer with $MgCl_2$), 0.25 µl of forward SSR primer at 20 pmol/µl and 0.25 µl of reverse SSR primer at 20 pmol/µl [44].

PCR products were resolved on an ABI-3130XL sequencer and analyzed using GeneMapper version 4.0 (Applied Biosystems). Microsatellite polymorphisms (chimerism in particular) were checked using Sequi-Gen GT Sequencing Cell electrophoresis (BIO-RAD) by silver staining technique [40, 44].

AFLP, SAMPL and M-AFLP Analysis

In order to detect intravarietal genetic variability, AFLP, SAMPL and M-AFLP molecular markers were used [39]. Analyses were performed by Starlet liquid handling workstations (Hamilton), ABI-3130XL capillary sequencer (Applied Biosystems) and ALFexpress-II semi-automated gel sequencer (Amersham). Restriction–ligation and preamplification were similar for the three marker types. Genomic DNA (500 ng) was digested and ligation was performed in 4 h at 37°C using *Eco*RI (or *Pst*I) and *Mse*I enzymes (5 U each), 1 U of T4 ligase, 50 pmol of *Mse*I-adaptor, 5 pmol of *Eco*RI-adaptor (or *Pst*I) in 1× RL buffer (20 mM Tris–acetate, 20 mM magnesium acetate, 100 mM potassium acetate, 5 mM DTT, 2.5 mg BSA) added with ATP to a final concentration of 10 mM [36]. Then, 5 µl of seven-fold diluted digested and ligated DNA was preamplified in 20 µl reaction mixture containing 75 ng of *Eco*RI + N (or *Pst*I + N) and *Mse*I + N primers, 1× PCR buffer (50 mM MgCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl), 10 mM dNTPs and 1 U of *Taq* DNA polymerase [39]. The *Eco*, *Pst* and *Mse* primers were called in tables and figures as E, P and M, respectively. The cycling conditions were 1 cycle of 45 s at 94°C, 30 s at 65°C, 1 min at 72°C and a touch-down profile (13 cycles with −0.7°C/cycle) for the annealing temperature and finally by an extension cycle of 5 min at 72°C [45, 46].

Amplified fragment length polymorphism (AFLP) markers analysis was performed with a Cy5 labelled *Eco*RI + 3 (or *Pst*I + 2) primer and an unlabelled *Mse*I primer using a GeneAmp PCR System 9700 (Applied Biosystems) [39]. Each 20 µl PCR contained 0.5 µl of the preamplified DNA, 50 ng of labelled *Eco*RI or *Pst*I primer, 30 ng of unlabelled *Mse*I primer, 2 µl of PCR buffer, 4 mM dNTPs and 0.4 U of *Taq* DNA polymerase [46].

Selective amplification of microsatellite polymorphic loci (SAMPL) markers analysis was performed using a procedure identical to that adopted for AFLP reported above, except for the second amplification, where the PCR was performed with the Cy5 labelled *As1* or *As2* adjacent-microsatellite primer and a standard *Mse*I primer with three selective nucleotides [47, 48]. The cycling conditions were 2 cycles of 45 s at 94°C, 30 s at 56°C, 1 min at 72°C, 13 cycles of 30 s at 94°C, 30 s at 55.3°C and 1 min at 72°C with a touch-down profile for the annealing temperature (13 cycles with −0.7°C/cycle) and 18 cycles of 30 s at 94°C, 30 s at 48°C and 1 min at 72°C; finally by an extension cycle of 5 min at 72°C [39].

Microsatellites amplified fragment length polymorphism (M-AFLP) markers analysis was performed using an AFLP procedure except for one of the primer used in the second amplification. The PCR was performed with the Cy5 labelled SSR or ISSR primer and a standard *Mse*I + 3 primer [36].

AFLP, M-AFLP and SAMPL primer combinations were carried out using the same amplification condition described above. After Cy5 PCR, 5 µl of FD loading buffer (98% formamide, 10 mM EDTA, 0.005% each of xylene–cyanol and bromophenol-blue) was added to each tube. Samples were denatured at 90°C for 5 min and then immediately

placed on ice. The amplification products of these three molecular marker types were resolved on ReproGelTM High Resolution pre-made acrylamide–bisacrylamide solutions (8% w/v) in modified TBE buffer (0.1 M Tris–HCl, 83 mM boric acid and 1 mM EDTA) and detected on a semi-automated DNA sequencer, the ALFexpress-II DNA Analysis System by Amersham Pharmacia Biotech [39]. The ladder used as internal standard was a mixture consisting of 100, 150, 200, 250 bp DNA fragments (Amersham Pharmacia Biotech) diluted to a final concentration of 4 femtomoles each. The 50–500 bp ladder was used at a final concentration of 0.7 femtomoles and 5 µl of this stock was loaded onto the gel. Gels were run for 450 min at 1300 V, 60 mA, 35 W and the temperature was maintained constant at 55°C. Markers were visualized automatically by the ALF-win Fragment Analyses 1.09 software [39]. Twenty accessions were analysed twice and ten for three times (DNA restriction, pre-amplification and selective PCR) to test the reproducibility of the molecular profiles; in every single experiment some genotypes (3 or 4) were analysed twice and carried out in two near lines (CAN-13 in Fig. 2).

Data Analysis

A binary presence (1) or absence (0) matrix was created for all DNA markers and Garnacha accessions, with each "locus" defined by a particular band size as identified by comparing sample lanes with known DNA ladders. Genetic similarity (GS) estimates among individuals were calculated in all possible pair-wise comparisons using the Dice's (1945) coefficient [49]. Dice's genetic similarity estimates between individuals, based on the probability that a marker from one accession will also be present in another, was calculated using the following formula: $GS_{ij} = 2a/(2a + b+c)$, where a represents the number of shared amplification products scored between the pair of samples/fingerprints (i and j) considered, b is the number of products present in i but absent in j, c is the number of products present in j but absent in i and d is the number of product absent either in i that in j. Thus, $GS_{ij} = 1$ indicates identity between i and j, whereas $GS_{ij} = 0$ indicates complete diversity. GS was calculated within (GS_W) and between (GS_B) the three Garnacha provenance (Italy, Spain and France) and marker systems (AFLP, SAMPL and M-AFLP), and also considering only the Italian accessions.

The cluster analysis was performed according to the unweighted pair-group arithmetic average method (UPGMA) and the dendrogram of all individuals was constructed from the symmetrical GS Dice's matrix.

Centroids of the 53 Garnacha accessions were bidimensionally plotted according to the principal coordinates extracted from the GS matrices estimated by the three molecular marker systems. All calculations and analyses were conducted using the appropriate routines of the NTSYS Version 2.10 software [50]. The information content of each marker system in discriminating Garnacha accessions was calculated using the marker index MI [51].

Results and Discussion

In order to verify the varietal identity, the analyses based on 14 SSR loci confirmed that only one SSR profile was obtained for the 53 accessions (Table 2). The presence of an additional allele at ISV3 locus (151 bp) was found in four Tocai rosso accessions and in a Sardinian Cannonau clone, which falls into a chimeric condition [52].

In order to study the intravarietal genetic variability of these materials, AFLP, SAMPL and M-AFLP analyses were also performed. The primer combinations are indicated in Table 3.

A total of 2,391 reproducible amplification products were obtained with the three molecular marker systems: 795 AFLPs, 608 SAMPLs and 988 M-AFLPs. Of these, 1,036 (43.3%) were polymorphic: 199 AFLPs, 342 SAMPL and 495 M-AFLPs. The analyses were repeated twice or more and the duplicate DNA samples analysed in every experiment confirmed the molecular approach reproducibility. The detected AFLP, SAMPL and M-AFLP polymorphisms were confirmed by these repeated analyses since the molecular markers reproducibility was about 100% also using different techniques (PAA gels with silver staining, gels High Resolution for ALFexpress-II sequencer, 3130XL Applied Biosystems capillary sequencer with five fluorescences).

The average number of marker loci assayed per single experiment was 49.7, 50.7 and 61.8, for AFLP, SAMPL and M-AFLP markers, respectively. The SAMPL molecular polymorphisms of 10 Sardinian Cannonau accessions (CAN-09/18, Table 1) and six French Grenache genotypes (GRE-30/34, Table 1) are shown in Fig. 2 by As2/Mse + AGA primers combination and silver staining technique. The line from 09 to 12 corresponding to Cannonau of Jerzu (CAN-09/12) with a specific amplification product, the same origin-specific amplification products are shown for Italian Cannonau and French Grenache genotypes (Fig. 2).

GS matrices were constructed for each possible pairwise comparison of individual genotypes within and between the three molecular marker systems by using Dice similarity coefficients (Table 4). Genetic similarity by AFLP markers generally agreed with the results of ampelographic analyses when the number of morphological characteristics considered was high [21] but this agreement fades when ampelographic analyses are based on a few characteristics

Table 2 Microsatellite profile of Garnacha tinta at 14 SSR loci and three reference varieties as Cabernet Sauvignon, Chardonnay and Moscato bianco

Loci SSR	Garnacha tinta		Cabernet Sauvignon		Chardonnay		Moscato bianco	
VVS2	137	145	139	151	137	143	133	133
VVMD5	226	240	232	240	234	238	228	236
VVMD7	239	243	239	239	239	243	233	249
VVMD24	212	218	210	219	210	218	214	219
VVMD26	249	249	249	251	249	255	251	251
VVMD27	194	194	175	189	181	189	179	194
VVMD28	247	247	237	239	221	231	249	271
VrZAG62	187	187	187	193	187	195	185	195
VrZAG79	256	256	246	246	242	244	250	254
ISV2	137	151	141	165	143	151	141	143
ISV3	135	145	133	139	139	145	133	139
ISV4	187	191	169	191	177	197	169	187
VMCNG4b9	138	164	168	176	158	158	158	166
VMC2H9	117	123	119	123	117	117	121	123

For each SSR locus were reported the two microsatellite alleles in bp. The 53 Garnacha accessions showed the same SSR profile

Table 3 AFLP, SAMPL and M-AFLP primer combinations used the intravarietal genetic analyses of the 53 Garnacha accessions

AFLP	SAMPL	M-AFLP
E + ACC/M + CAT	As1/M + CAA	ISSR#10/M + ACA
E + ACT/M + CTT	As1/M + CGT	VVS2f/M + ATC
E + ATG/M + CAG	As1/M + CTC	VVMD5f/M + CAT
E + CAA/M + CAT	As1/M + CGG	ISSR#03/M + CTT
E + CAT/M + CCT	As1/M + AGT	P + AT/ISV3r
E + CGT/M + CTG	As1/M + ATT	E + CAG/ISV4r
E + CTG/M + CAA	As2/M + CTT	E + AGT/ISSR#08
E + ACT/M + AGG	As2/M + CAG	VrZAG62f/M + ATT
E + CGA/M + ATT	As2/M + CGA	VrZAG79f/M + CTA
P + AT/M + CAG	As2/M + CGT	VVMD28f/M + CAA
P + AT/M + CTG	As2/M + CTG	ISSR#10/M + CAG
P + AG/M + CGT	As2/M + AGA	VVMD/f/M + ATG
P + AG/M + CCT		ISV4f/M + CGT
P + AA/M + CTT		VVMD27f/M + AGG
P + AT/M + CAA		VMCNG4b9f/M + CTG
P + AG/M + CTA		ISSR#07/M + AGG

E, *P* and *M* are the Eco-RI, Pst-I and Mse-I AFLP primers respectively with the 2 or 3 selective nucleotides (A, G, C and T), As1 and As2 SAMPL primers, while M-AFLPs were the SSR/ISSR and AFLP primer combinations

[53]. The SAMPL and M-AFLP analyses allowed to distinguish Garnacha tinta and Garnacha blanca accessions (Figs. 1, 3). In Fig. 1, the two genotypes of G. blanca are shown the same AFLP molecular profile (GAR-36 and GAR-46 are in the line 36 and 46, respectively) and in the dendrogram are grouped in the same cluster.

Table 4 Statistics related to the 53 Garnacha accessions showed for marker type, where "*n*" is the number of detected loci, "*p*" is the number of polymorphic and "*m*" of monomorphic loci, "% lp" is the percentage of polymorphic loci, GS_W and GS_B are the Dice's mean genetic similarities within and between the three marker types, "GS_{TOT}" is the total genetic similarity and "MI" is the marker index according Powell et al. [51]

Primer	*n*	*p*	*m*	% Ip	GS_W	GS_B	GS_{TOT}	MI
AFLP	795	199	596	25.0	0.9708	0.9610	0.9692	5.980
SAMPL	608	342	266	56.3	0.9617	0.9533	0.9572	6.522
M-AFLP	988	495	493	50.1	0.9318	0.9266	0.9302	9.941
Tot	2,391	1,036	1,355	43.3				

Genetic similarity (Dice) estimated within and between the three molecular marker types (AFLP, SAMPL, M-AFLP) was reported in Table 5. The GS_{TOT} values show that the M-AFLP molecular markers were the most efficient in discriminating the 53 Garnacha genotypes: AFLPs also show many monomorphic markers (25%, Fig. 1; Table 4), SAMPLs distinguished more genotypes on the basis of their geographic origin (Sardinia, Sicily, Northern Italy, France, Spain; Fig. 2) and M-AFLPs revealed many plant-specific polymorphims, differentiating all accessions (lower value of GS_{TOT}). Computation of the MI parameter revealed a marker system utility of M-AFLPs (9.941) much higher than those of SAMPL (6.522) and AFLPs (5.890), as a consequence of their ability to identify multiple polymorphisms.

Genetic similarity estimated within (GS_W) and between (GS_B) the three different origin (Italy, Spain and France) and for the four Italian geographic areas (Sicily, Sardinia,

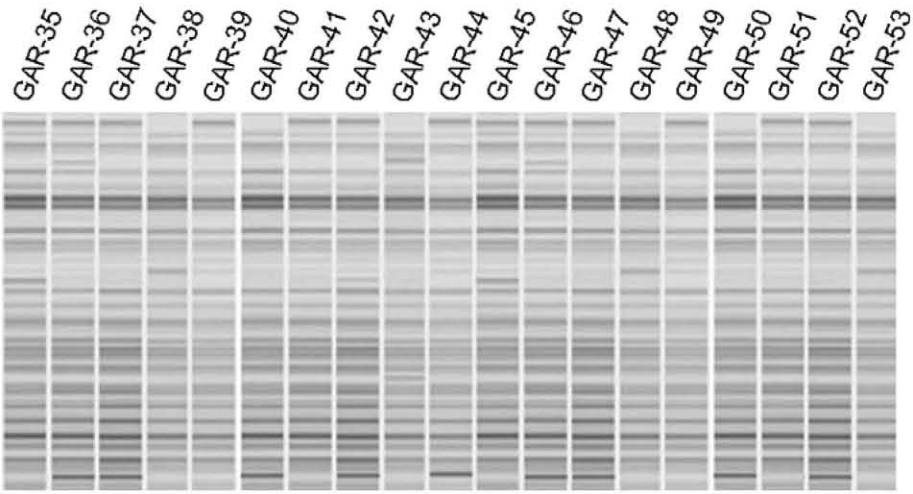

Fig. 1 Example of a digitalized electropherogram of the AFLP profiles obtained for the 19 Spain Garnacha (GAR) accessions (from GAR-35 to GAR-53 lines) using an ALFexpress-II DNA Automated Sequencer (Eco + CGA/Mse + ATT primers combination). The majority of AFLP markers were monomorphic but there were some clear differences: the line 36 and 46 are very similar (two G. blanca genotypes) and line 37 and 49 showed only a different marker (two G. peluda genotypes). Genotypes from left to right: 35 = Garnacha tinta, 36 = G. blanca, 37 = G. peluda, 38 = G. roja; from 39 to 45 = Garnacha tinta; 46 = G. blanca, 47 = G. erguida, 48 = G. roya, 49 = G. peluda; from 50 to 53 = G. tinta (Table 1)

Table 5 Genetic similarity estimates according to Dice coefficient calculated within (GS_W) and between (GS_B) the three geographic origins (Italy, France and Spain) using AFLP, SAMPL and M-AFLP molecular markers, particularly within the Italian materials (Alicante, Cannonau, gamay perugino and Tocai rosso accessions)

	n	Dice's genetic similarity	
		GS_W	GS_B
Italy	28	0.9481	0.9480
France	6	0.9662	0.9457
Spain	19	0.9862	0.9315
Tot	53		
Italy			
Alicante	8	0.9755	0.9312
Cannonau	10	0.9647	0.9332
Gamay perugino	4	0.9625	0.9383
Tocai rosso	6	0.9692	0.9337

Umbria and Tuscany, Colli Berici) is reported in Table 5. Italian samples have shown a high genetic variability within genotypes ($GS_W = 0.9481$) while Spanish samples have shown a high genetic similarity ($GS_W = 0.9872$). Considering the Italian accessions, the GS_W (0.9481) is very similar to GS_B (0.9480), but the four Italian origins (or Garnacha accession names) are clearly separated by these molecular markers (Figs. 3, 4; Alicante from Sicily, Tocai rosso from Colli Berici of Vicenza, Cannonau from Sardinia, Gamay perugino from Umbria).

Dice's GS matrix was used to perform the cluster analysis: similarity relationships among Garnacha accessions are reported in Fig. 3. The UPGMA dendrogram as defined by AFLP, SAMPL and M-AFLP markers displayed the 53 genotypes clustered into two distinct groups: all individuals of Cannonau (CAN) from Sardinia (Italy) were positioned in the first group and all remainder accessions, clustered into distinct subgroups, in the second one; all accessions of Alicante (ALI) and Gamay perugino (GAM) from Italy were positioned in the first subgroup, Spanish Garnacha (GAR) and Italian Tocai rosso (TOR) accessions in the second one, and French Grenache (GRE) in the third subgroup. All 53 genotypes were hence separated on the base of their zone of cultivation (Sardinia, Sicily, Umbria, Vicenza province, Spain, France) therefore also according to the different accessions namely Cannonau, Alicante, Gamay perugino, Tocai rosso, Garnacha cvs and Grenache, respectively.

GS matrices estimates and dendrogram results were in agreement with Principal Coordinate Analysis reported in Fig. 4. The bi-dimensional plotting of centroids is shown six different groups: (1) Italian Alicante accessions from Sicily; (2) Italian Tocai rosso accessions from Vicenza area (Colli Berici); (3) Italian Gamay perugino accessions from Tuscany and Umbria, (4) Spanish Garnacha accessions from Andalucia, Aragón, Cataluña, Castilla y León, Madrid; (5) French Grenache accessions; (6) Italian Cannonau accessions from Sardinia. The first coordinate allowed to distinguish clearly Spanish, French and Italian accessions instead the second one separated the Italian origins, therefore the Italian accession names which Alicante, Tocai rosso, Gamay perugino and Cannonau. The PCA analysis confirmed the high genetic variability within Italian genotypes in agreement with the four different

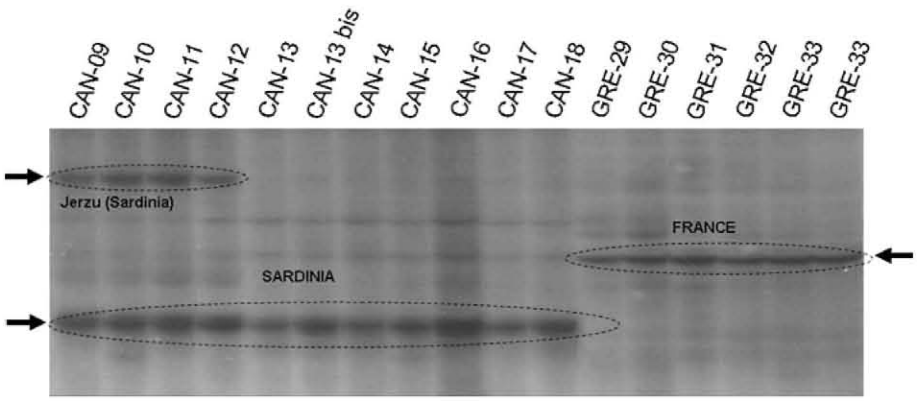

Fig. 2 Example (2 *arrows*, Sardinia and France) of geographic origin-specific polymorphisms by SAMPL molecular markers of Sardinian Cannonau (from CAN-09 to CAN-18 lines) and French Grenache (from GRE-29 to GRE-33 lines) accessions using the Silver Staining technique. The lines from CAN-09 to CAN-12 corresponding exactly to Sardinian Cannonau from Jerzu (arrow) with a specific amplification product. The sample CAN-13, for example, was analysed twice (CAN-13 bis) repeating DNA extraction, restriction, pre-amplification and selective PCR to test the reproducibility of the molecular approach

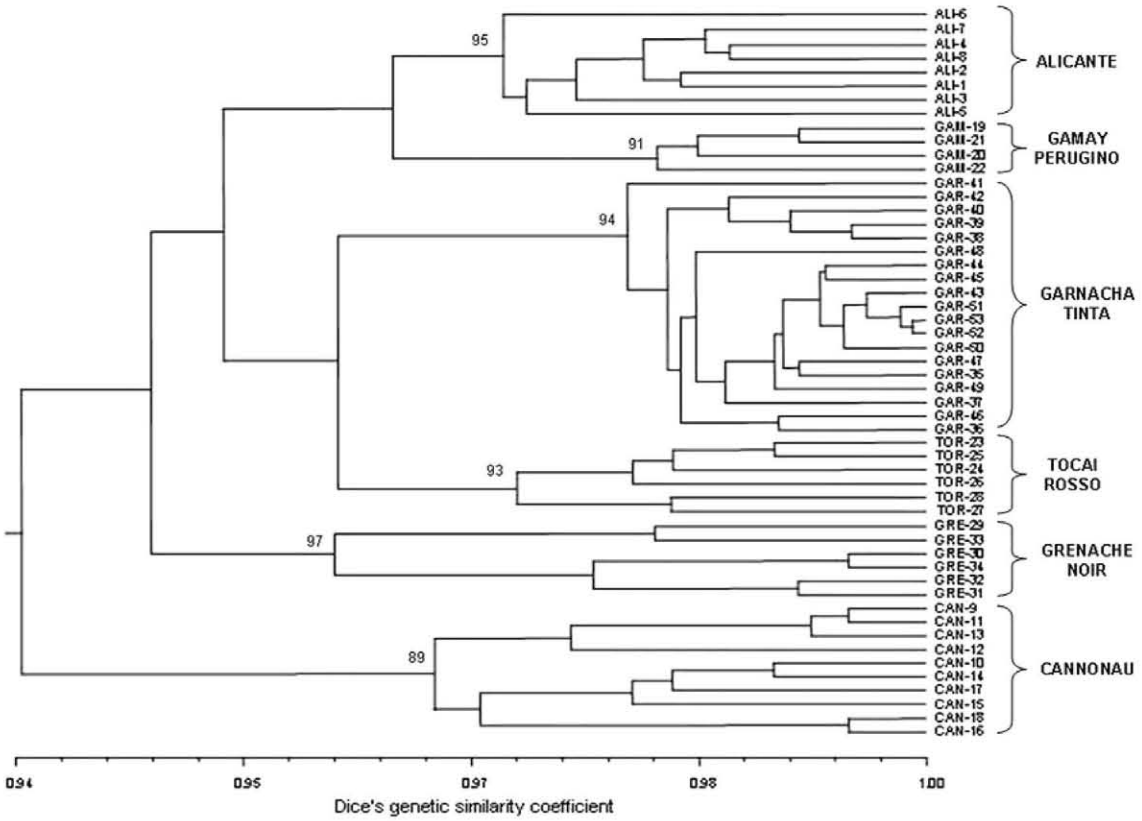

Fig. 3 Dendrogram of 53 Garnacha accessions based on Dice's Genetic Similarity (Coph. $V. = 0.872$). The genotypes were clustered according their geographic origins and accession names

geographic origin, on the contrary the 19 Spanish accessions are clustered in a homogeneous group that is shown a high genetic similarity ($GS_W = 0.9872$) (Table 4).

The most important result into the centroids was the clean separation among the three geographic origins (Italy, Spain and France) and also the high genetic distance

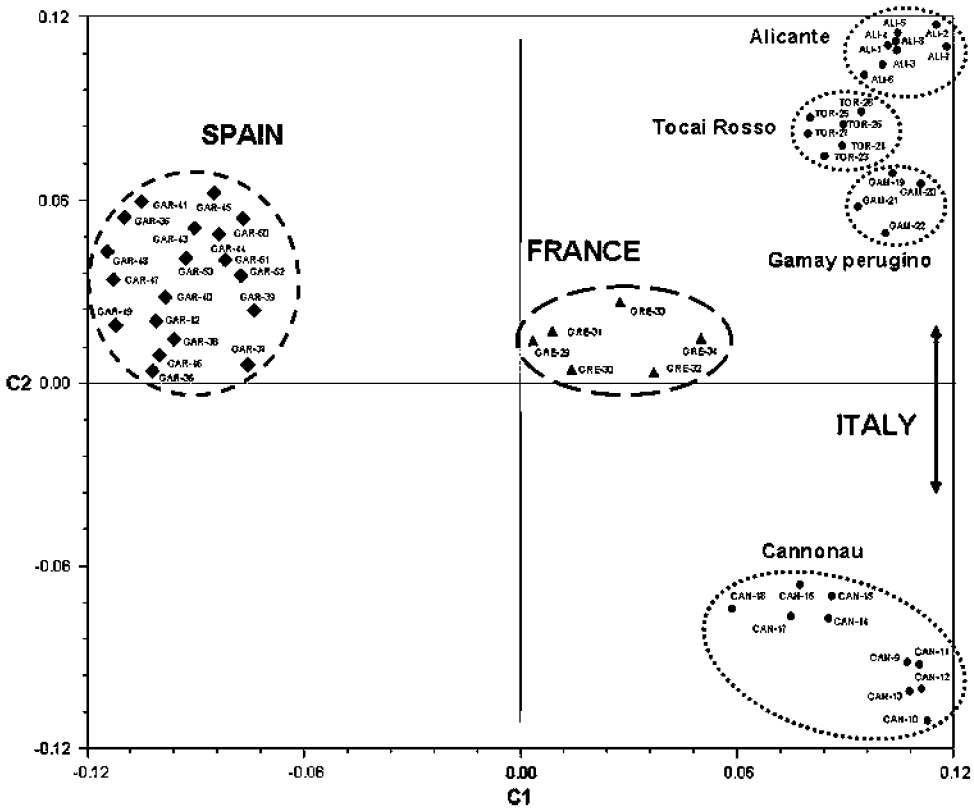

Fig. 4 Centroids of 53 Garnacha accessions based on Dice's Genetic Similarity showed clearly six different groups which Alicante accessions from Sicily, Tocai rosso from Vicenza area, Gamay perugino from Tuscany, Cannonau from Sardinia (Italian accessions), Grenache samples from France and Garnacha genotypes from Spain

among the Italian accession from different zones of cultivation (Alicante, Tocai rosso, Gamay perugino, Cannonau). The 19 Spanish accessions from six different Garnacha varieties are grouped in the same cluster, well separated to the Italian and French accessions.

Conclusions

The results of the microsatellite analysis confirmed that all 53 Garnacha accessions (Table 1) were of the same cultivar, even if they were called Garnacha tinta in Spain, Grenache noir in France and Cannonao or Tocai Rosso or Alicante in Italy, with other local names as Gamay perugino in Tuscany and Umbria or Cannonau in Sardinia. The presence of an additional SSR allele (chimeric state) allowed for discrimination of four Tocai rosso (TOR-23/26) and one Cannonau (CAN-13) accessions [41, 54].

The three molecular marker systems, AFLP, SAMPL and M-AFLP, were able to clearly distinguish the 53 Garnacha accessions from Italy, Spain and France. The large number of molecular markers and their high degree of polymorphism make them important tools for many genetic studies. These results confirm the ability of M-AFLP and SAMPL markers to discriminate all accessions by theirs plant-specific molecular markers. This technique provides an excellent means of Garnacha clones identification and fingerprinting and should play a major role in the efficient management of *Vitis* germplasm collections. This work provides an important molecular contribute to study the clones genetic variability and identification. The high molecular variability of Italian materials and the high genetic similarity among Spanish accessions suggest that the hypothesis of the Italian origin of this cultivar can be considered.

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